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Serologically Silent Hepatitis B Virus Coinfection in Patients With Hepatitis C Virus-Associated Chronic Liver Disease: Clinical and Virological Significance

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Frequent coinfection of surface antigen-negative hepatitis B virus (silent HBV) in hepatitis C virus (HCV)-associated chronic liver disease (CLD) has been reported. The clinical and virological significance of silent HBV infection was investigated in 65 patients with HCV-associated CLD who subsequently received interferon (IFN) therapy. HBV DNA was detected in 34 (52.3%) patients by a nested polymerase chain reaction (PCR). Virologically, all of the 34 patients were found to have HBV with an eight-nucleotide deletion in the core promoter. Coinfection of silent HBV was more frequent with HCV genotype 1b than in 2a (64.3% vs 28.6%, $P < .01$). With HCV genotype 1b, the serum RNA level was significantly higher ($\geq 10^6$ copies per milliliter vs $\leq 10^5$ copies per milliliter) in patients with silent HBV than those without coinfection ($P < .01$). Clinically, silent HBV was associated with a higher level of serum alanine aminotransferase (158.5 ± 104.8 vs 121.8 ± 78.6 IU/l; mean ± SD) and a greater histological activity of hepatitis as evaluated by histological activity index score (9.4 ± 3.8 vs 8.6 ± 4.5; mean ± SD), although it was not statistically significant. Silent HBV was also associated with poor efficacy of IFN therapy ($P < .01$). The results suggest that silent HBV has some promoting effect for HCV replication, at least for HCV genotype 1b, and may affect the histological activity of hepatitis and IFN response in HCV-associated CLD. ***J. Med. Virol. 58: 201–207, 1999.***

KEY WORDS: serologically silent HBV; hepatitis C virus-associated chronic liver disease; interferon therapy; nested polymerase chain reaction

INTRODUCTION

Hepatitis B virus (HBV) shows a unique persistent infection, known as a serologically silent infection, characterized by the positivity of serum HBV DNA as seen by a nested polymerase chain reaction (PCR), despite the absence of serum hepatitis B surface antigen (HBsAg) [Repp et al., 1992; Uchida et al., 1994; Chung et al., 1995; Feitelson et al., 1995; Fukuda et al., 1996]. Several reports have shown that this serologically silent HBV coinfects with hepatitis C virus (HCV) in patients with HCV-associated chronic liver disease (CLD) with variable frequency (50–87%) [Gonzalez et al., 1995; Villa et al., 1995; Uchida et al., 1997; Zignego et al., 1997; Koike et al., 1998]. Because the prevalence of serum HBsAg-positive HBV in patients with HCV-associated CLD has been reported to be less than 20% [Chen et al., 1990; Fong et al., 1991; Ohkawa et al., 1994, 1995], a high prevalence of coinfection with silent HBV suggests that the coinfected silent HBV has some implication in the pathogenesis of HCV-associated CLD.

The clinical significance, however, of silent HBV coinfection in HCV-associated CLD has not been fully understood because of the limited number of studies [Villa et al., 1995; Zignego et al., 1997]. Moreover, although Uchida et al. [1997] showed that the silent HBV coinfected with HCV is a mutant HBV that has an 8-nucleotide (nt) deletion in the core promoter sequence, this interesting finding has not been confirmed using a larger number of patients. To elucidate the clinicopathological significance of silent HBV coinfection in HCV-associated CLD, in the present study, we investigated the relationship between the coinfection and HCV amount, HCV genotype, and the clinical ef-

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ficacy of interferon (IFN) therapy, as well as the prevalence of silent HBV in 65 patients with HCV-associated with CLD. In addition, we investigated the core promoter sequence in HBV identified in sera from these patients. Using this method, the prevalence of the 8-nt deletion mutant HBV in the coinfecting HBV was also investigated.

Materials and Methods

Patients and IFN Therapy

Sixty-five patients with HCV-associated CLD participated in this study. All of the patients were followed up at the University Hospital of Shimane Medical University. All the patients had anti-HCV antibody (second-generation, ORTH Diagnostic Laboratory, Tokyo) and HCV RNA in serum. All patients subsequently received IFN treatment at the University Hospital. The schedule for IFN treatment was standardized as follows; a 6 megaunit of recombinant IFN-α2b (Intron A®, Shering Plough, Kenilworty, NJ) by intramuscular injection daily for 2 weeks, followed by 3 times a week for 22 weeks. Laboratory tests were performed weekly during the first 2 weeks, biweekly during the remainder of IFN treatment, and then monthly for 6 months after the treatment was completed. Patients were classified into two categories, IFN responders and nonresponders, on the basis of the clinical course. Responders (20 in this group) were defined as patients whose alanine aminotransferase (ALT) levels in serum decreased to the normal range (<40 IU/l) during treatment, along with the disappearance of serum HCV RNA, and remained at a normal level for 1 year after treatment. Nonresponders (45 patients) had ALT levels persistently above the normal range during and after IFN treatment. HCV RNA was detected at the end of the IFN treatment in nonresponders. The tests of patients whose ALT levels declined initially to the normal range during IFN treatment, but again increased above the normal range within 6 months after treatment, were positive for HCV RNA at the end of the IFN treatment; these patients were classified as nonresponders. As a positive control, sera from 10 patients with hepatitis B e antigen (HBeAg)-positive chronic hepatitis B and 5 patients with anti-HBe were examined. Sera from 20 healthy volunteers were also used as a negative control.

Serological and Virological Markers of HBV and HCV Infection

Serum HBsAg (Radioimmunoassay, Abbott Laboratories, Chicago, IL), anti-HBs (Radioimmunoassay, Abbott), anti-HBc (Radioimmunoassay, Abbott) and anti-HCV (second-generation, Dainabott, Tokyo) were tested.

DNA Extraction From the Serum

Serum taken before IFN therapy was analyzed for HBV DNA. The DNA was extracted from 100 to 300 μl of serum using phenol chloroform/isoamylalcohol after proteinase K digestion as reported previously [Okamoto et al., 1990] and recovered by ethanol precipitation in the presence of 1 μl of glycogen (Sigma Chemical Campany, St. Louis, MO). Finally, the DNA was dissolved in 20 μl of 10 mM Tris HCL (pH 8.0), 1 mM EDTA, and stored at −20°C until PCR occurs.

Nested PCR for Core Promoter Region of HBV DNA

HBV DNA of a 176 base-pair length, including the core promoter region, was amplified by nested PCR, using 5 μl of the DNA solution as a template. The first PCR primer pairs for nested PCR were p202 5′-CTGCCGTTCCGGCCGACCAC-3′ (sense, nt1373–1392) and H2 (antisense). The second PCR primer pairs were H1 5′-CATAAGAGGACTCTTGGACT-3′ (sense, nt1535–1674) and p201 5′-ATTAGGCAGAGGTGAAAAAG-3′ (antisense, nt1692–1711). The nucleotide numbers were based on a report by Fujiyama et al. [1983]. The PCR conditions were the same as those used in our previous study [Fukuda et al., 1996]. As a negative control of the PCR, distilled water was always used as a template. After the PCR, 5 μl of aliquot was electrophoresed in 1% agarose gel containing ethidium bromide and the existence of the target band was confirmed under an ultraviolet lamp. Positivity of HBV DNA was defined as having a DNA sequence of HBV DNA after sequencing.

DNA Sequencing of the Core Promoter Region of HBV

The second PCR product, including the core promoter region, was ligated with the TA cloning vector using a TA cloning™ Kit (Invitrogen Co., Carlsbad, CA). After transformation of the competent cells by the ligated DNA, the plasmid DNA was extracted from the transformed cells by an alkaline lysis method [Sambrook et al., 1989] using a QIAprep Miniprep Kit (QIAGEN Inc. Valencia, CA). DNA sequencing was determined by a dideoxy chain termination method using a BcaBEST™ Dideoxy Sequencing Kit (TAKARA Shuzo, Osaka Japan) in 5 to 10 clones from each patient. The sequence primers were the same as for the nested PCR of the core promoter region.

Determination of Genotype and Amount of HCV RNA

The genotype of HCV RNA was determined according to the classification of Simmonds et al. [1993] consistent with the nucleotide sequence variability in the 5′ noncoding region of HCV as previously reported. HCV RNA in the serum was quantified by a competitive reverse transcription-PCR according to the previous method described by Hagiwara et al. [1993]. The amount of HCV RNA was expressed as copy numbers in serum (10^N copies per milliliter).

Histological Examination

A liver biopsy was carried out before IFN therapy for all of the patients. Liver specimens were fixed in 10% formalin, followed by paraffin-embedding, and 4 μm-

TABLE I. Subpopulation of HBV in Serum From Patients With Chronic Hepatitis B and HCV-Associated CLD*

Group	No. of patients[a]	Clone wild/mutant[b]	Prevalence of mutant clone (%)
HCV-CLD $n = 65$	34	42/146	100
CH-B		50/0	0
HBeAg(+) $n = 10$	10	38/0	0
anti-HBe $n = 5$	5	25/0	20
Control[c] $n = 20$	0	—	—

*The prevalence of mutant HBV with a core promoter mutation was investigated by DNA sequencing.
HCV-CLD, hepatitis C virus-associated chronic liver disease; CH-B, chronic hepatitis B.
[a]Number of patients positive for HBV DNA by PCR.
[b]Number of clones (clones with wild sequence/clones with deletion at the core promoter).
[c]Healthy volunteers.

TABLE II. Relationship Between Silent HBV Infection and HBV Markers in Serum and Liver*

HBV marker	HBsAb + HBcAb + $n = 8$	HBsAb + HBcAb – $n = 7$	HBsAb – HBcAb + $n = 10$	HBsAb – HBcAb – $n = 40$
Serum DNA Positive	4	2	8	20
HBsAg	2	0	2	8
HBcAg	0	0	0	0
Serum DNA Negative	4	5	2	20
HBsAg	0	0	0	0
HBcAg	0	0	0	0

*Immunohistochemical study in the liver. HBsAb, anti-HBs; HBcAg, anti-HBc; +, positive; –, negative (in serum).

thick sections were processed for hematoxylin and eosin staining, and indirect immunoperoxydase staining. The histological activity of hepatitis was evaluated using a histological activity index (HAI) score system [Knodell et al., 1981].

Immunohistochemical Study

HBsAg and hepatitis B core antigens (HBcAg) in the liver were investigated by immunostaining. Liver sections were deparafinized and reacted with the first antibody (anti-HBs mouse monoclonal antibody and anti-HBc rabbit monoclonal antibody. DAKO Japan Co.Ltd, Tokyo) at 4°C overnight. The sections were then treated with a second antibody (gout anti-mouse and anti-rabbit IgG conjugated horseradish peroxydase, DAKO) at 37°C for 1 hour. The reaction products were visualized by treatment with 3,3′-diaminobenzine tetrahydrochloride. As a control, immunostaining using normal mouse and rabbit serum, as for the first antibody, was also carried out.

Statistical Analysis

The statistical significance in the difference of the patients' age, gender, serum ALT level, and the HAI score between patients, with and without coinfection of silent HBV, were assessed by a Student's t test. The relationship between silent HBV coinfection and the HCV RNA genotype, serum RNA level, and the IFN efficacy was assessed by a χ^2 test. In each test, the value of significance was .05.

Results

Prevalence of HBV DNA in Serum

None of the 65 patients exhibited a positive PCR result at the first PCR, while HBV DNA was detected in 34 (52.3%) patients by the second PCR (Table I). In patients with chronic hepatitis B, HBV DNA was detected in all of the 10 HBeAg-positive patients by the first PCR and 5 anti-HBe positive patients were found by the second PCR. HBV DNA was not detected in the 20 healthy volunteers, even at the second PCR. None of the patients with HBeAg-positive chronic hepatitis B, nor the healthy volunteers, were positive for HCV RNA.

HBV Markers in Serum and Liver in Patients With Chronic Hepatitis C

HBsAg was not found in any of the 65 patients, while anti-HBs and anti-HBc were positive in 15 (23.1%) and 18 (27.7%) patients, respectively. The relationship between silent HBV infection and markers of HBV infection is shown in Table II. Sixty percent of the patients who had either or both anti-HBs and anti-HBc also had silent HBV infection. However, about half (20/41) of the patients who did not exhibit any serological markers of HBV infection also had silent HBV infection. HBcAg was not detected by immunochemistry, but a weak staining of HBsAg was found in 12 of the patients with silent HBV infection (data not shown).

Subpopulation of HBV Classified According to the Core Promoter Sequence

The DNA sequence of the core promoter was investigated in 188 clones amplified from the 34 patients with a positive PCR result (Table I). All 34 patients had the 8-nt deletion (nt1640–1647) mutant clone (Fig. 1). Coexistence of wild-type clones was observed in 29.9% (10/34). Among these 10 patients, however, the population of wild clones varied from 37.5% to 80%. Two patients had 41 nt-deletion clones (nt1647–1682) together with eight 8-nt deletion clones. In patients with chronic hepatitis B, all clones (75 clones) were found to be without the deletion regardless of the state of HBe/anti-HBe in serum.

Clinical and Pathological Difference of Patients With and Without Coinfection of Silent HBV

Clinically, there were no distinct features of the patients with silent HBV coinfection (Table III). The prevalence of HBV was not different between those with chronic hepatitis and those with early liver cirrhosis. Although the serum ALT levels and histological hepatitis activity as evaluated by the HAI score were higher in the coinfected patients (158.5 ± 104.8 vs 121.8 ± 78.6 IU/l, 9.4 ± 3.8 vs 8.6 ± 4.5, respectively), it was not statistically significant. IFN response was unfavor-

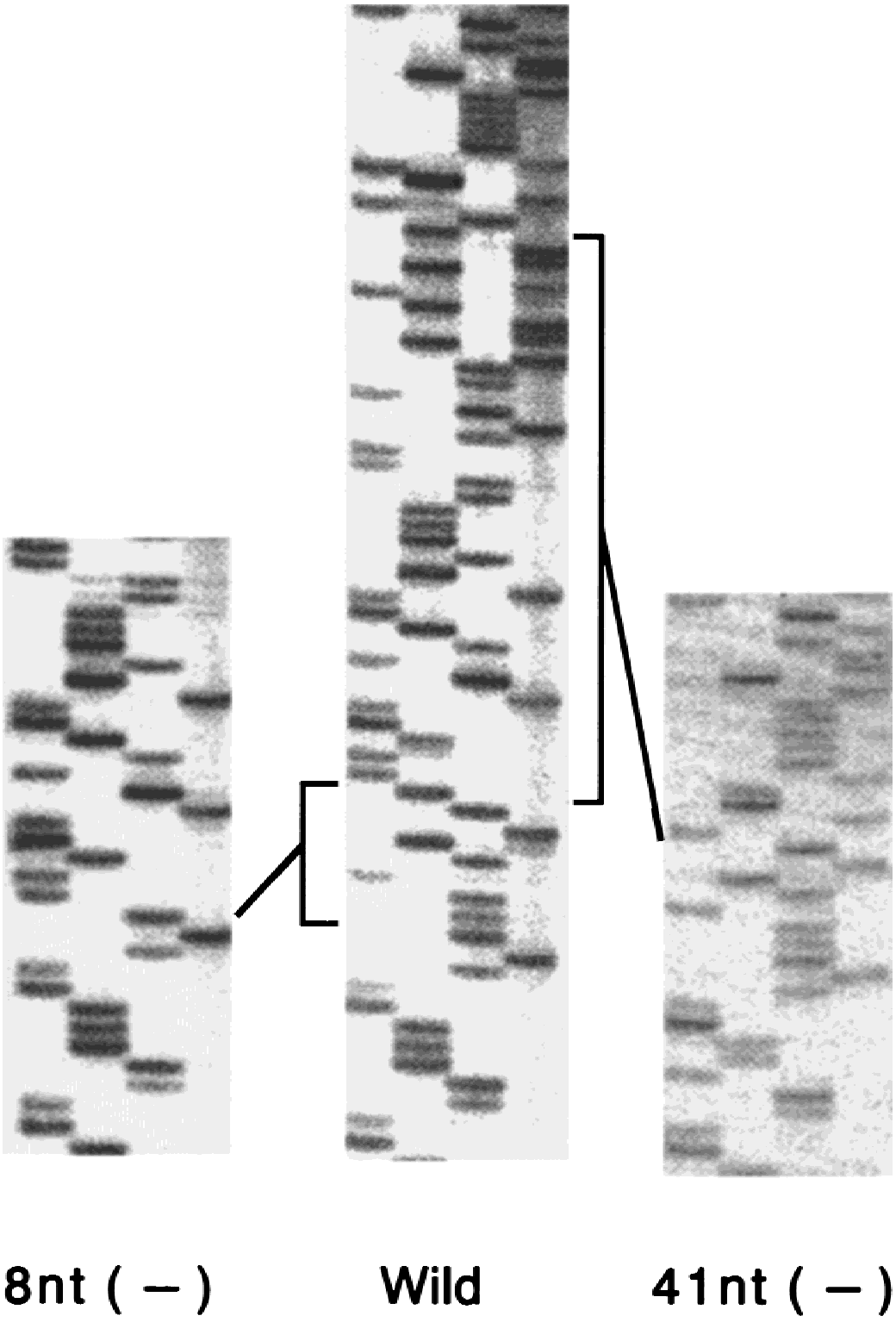

Fig. 1. Core promoter sequences identified in serologically silent HBV-infected sera. An 8-nt deletion (nt1640–1647) was detected in HBV amplified from all of the silent HBV-positive patients [8 nt(–)]. Coexistence of the wild-type sequence [Wild] was seen in 10 patients. Two patients had a 41-nt deletion mutant clone [41 nt (–)] together with the 8-nt deletion mutant.

TABLE III. Clinical Features of Patients With and Without Silent HBV Coinfection in HCV-Associated CLD

Characteristics	Silent HBV[a] (+)	Silent HBV (–)
No. of patients	34	31
Gender (Male/female)	24/10	14/17
Age (yr, mean ± SD)	50.9 ± 12.0	51.8 ± 9.3
Blood transfusion[b]	11	8
Liver histology		
Chronic hepatitis	29	28
Early cirrhosis	5	3
ALT[c] (IU/l; mean ± SD)	158.5 ± 104.8	121.8 ± 78.6
HAI score[d] (mean ± SD)	9.4 ± 3.8	8.6 ± 4.5
IFN response		
Responders	$4^{P < .01}$	16
Nonresponders	30	15

[a]HBsAg-negative HBV infection detected by nested PCR.
[b]History of blood transfusion.
[c]Alanine aminotransferase in serum.
[d]Histological activity index score.

able in patients with silent HBV coinfection when compared to those without silent HBV infection, and it was statistically significant ($P < .01$).

Virological Features of Patients With and Without Silent HBV Coinfection

The silent HBV coinfection rate was significantly frequent with HCV genotype 1b (27/42; 64.3%) as compared to HCV genotype 2a (6/21; 28.6%, $P < .01$) (Table IV). The serum HCV RNA level was higher in patients with silent HBV coinfection compared to those without silent HBV ($P < .01$). However, in the HCV genotype-specific analysis, this relationship did not reach a statistical value in HCV genotype 2a (Table V).

TABLE IV. Virological Characteristics in Patients With and Without Silent HBV Infection

Parameters	Silent HBV (+)[a] $n = 34$	Silent HBV (–) $n = 31$
HCV genotype		
1b ($n = 42$)	$27^{P<.01}$	15
2a ($n = 21$)	6	15
2b ($n = 2$)	1	1
Serum HCV RNA[b] level		
$10^{6\leq}$ ($n = 53$)	$32^{P<.01}$	21
$10^{5\geq}$ ($n = 12$)	2	10

[a]HBsAg-negative HBV infection detected by nested PCR.
[b]Copy number per milliliter of serum.

TABLE V. Relationship Between Silent HBV Infection and HCV RNA*

HCV genotype[a]	Serum level of HCV RNA[b] $\leq 10^5$	Serum level of HCV RNA[b] $\geq 10^6$	P[c]
1b			
Silent HBV[d]			
(+) $n = 27$	1	26	<0.05
(–) $n = 15$	4	11	
2a			
Silent HBV			
(+) $n = 6$	1	5	NS
(–) $n = 15$	5	10	
2b			
Silent HBV			
(+) $n = 1$	0	1	NS
(–) $n = 1$	1	0	

NS, not significant.
*Data are shown as the number of patients.
[a]Simmonds' classification.
[b]Copy number (copies/ml).
[c]χ^2-test.
[d]HBsAg-negative HBV infection detected by nested PCR.

Discussion

In the present study, over half of the patients with HCV-associated CLD were infected with silent HBV. Moreover, a characteristic core promoter mutant was identified in all patients with the coinfection, although a small number of them also had the wild-type core promoter HBV and other deletion mutants. This characteristic core promoter mutation has been reported in patients with non-B non-C chronic hepatitis [Uchida et al., 1995; Fukuda et al., 1996] and in anti-HBe positive asymptomatic HBV carriers [Fukuda et al., 1995], in whom the serum HBV level was extremely small. The low level replication of HBV in silent HBV infection does not seem to result from an interference by HCV [Shih et al., 1993; Koike et al., 1995] because silent HBV infection itself does not necessarily accompany HCV infection [Uchida et al., 1994; Chung et al., 1995; Fukuda et al., 1996]. As for the significance of the 8-nt deletion mutation, we propose the following explanation. The core promoter is located at the distal part of the X gene of HBV, which contains important elements for HBV replication, including the liver specific enhancer II [Wang et al., 1990], in addition to the core promoter. Many cellular transcriptional factors also bind to these elements [Lopez-Cabrera et al., 1990, 1991; Maguire et al., 1990]. The X protein has a trans-activating effect on both cellular and viral gene transcription by modifying the cellular transcriptional factors [Spaudau et al., 1988; Aufiero et al.,1990; Maguire et al., 1990; Unger et al., 1990; Rossner et al., 1992]. This trans-activating effect disappears by truncation of the X protein [Ritter et al., 1990]. The 8-nt deletion affects the core promoter binding site overlapping the liver specific enhancer II and truncates the X protein by 20 aa by creating a translational stop codon from the C-terminal end where an essential domain for the transactivation locates (aa 31–142) [Takada et al., 1990]. The distal part of the X gene is necessary to produce an infectious virus in a susceptible host [Chen et al., 1993; Zoulim et al., 1994]. Thus, the 8-nt deletion may be responsible for the extremely low level of viral replication, which could explain the seronegativity of HBV markers. HBsAg was observed only in a small portion of our patients, and HBcAg was not detected by immunostaining. The low prevalence of these antigens in the liver has been reported previously [Uchida et al., 1997]. As the prevalence of HBcAg was extremely low in that report, the absence of HBcAg in the present study may be due to the lower sensitivity of the immunostaining.

Why the mutant HBV, but not wild HBV, so frequently coinfected patients with HCV-associated CLD

is not clear. However, this mutant HBV seems to have some promoting effect on HCV replication, since Uchida et al. [1997] have reported that coinfection of the mutant HBV, but not wild HBV, accelerates the HCV replication in vitro. Because this coinfection was associated with a higher level of serum HCV RNA in the present study, this mutant HBV may accelerate the HCV replication also in vivo. Although coexistence of HBV without the core promoter mutation was also found in a small portion of patients, coexistence of the wild- and the mutant-type HBV has been reported in patients with silent HBV infection [Repp et al., 1992]. Thus, the mutant virus may be dominant-negative in HBV replication. Silent HBV coinfection is about three times more frequent in HCV genotype 1b than in genotype 2a. Moreover, this coinfection was associated with a higher level of serum HCV RNA in HCV genotype 1b, but not in HCV genotype 2a. Since the HCV RNA level is usually larger in HCV genotype 1b as compared with HCV genotype 2a [Kohara et al., 1995; Kobayashi et al., 1996], a higher prevalence of the coinfection with HCV genotype 1b may be reasonable. Since it has been suggested that viral replication is higher in genotype 1b than 2a [Tsukiyama et al., 1992; Kohara et al., 1995; Kobayashi et al., 1996], silent HBV coinfection may have some implication in the replication-efficiency of HCV genotype 1b. However, the false-negativity by PCR in patients infected with HCV genotype 2a must also be considered. These patients may be infected with smaller numbers of silent HBV, which is below the sensitivity of the nested PCR. A more sensitive PCR may reveal a higher prevalence of the mutant HBV in patients infected with HCV genotype 2a as well.

In general, dual or triple infection with hepatitis viruses leaves more severe hepatitis and less effective IFN response [Farci et al., 1994; Liaw et al., 1995; Weltman et al., 1995]. Zignego et al. [1997] reported that patients with coinfection of silent HBV were more resistant to IFN therapy, although the number of patients and the dose of IFN were small (14 cases and 3 megaunits/week for 12 months). Although we found that coinfection with silent HBV was associated with poor IFN efficacy in HCV-associated CLD even after a 6-month therapy, IFN nonresponders were also characterized by a larger level of HCV RNA and dominant infection with HCV genotype 1b, both of which are well-known predictive factors of poor IFN response [Tsubota et al., 1994]. Thus, it cannot be concluded that silent HBV coinfection affects the IFN response in patients with HCV-associated CLD. However, silent HBV coinfection may have some implication on IFN response by modulating the HCV replication efficacy. The exact mechanism requires further investigation.

Silent HBV coinfection is common in HCV-associated CLD. Silent HBV may have some promoting role for HCV replication, at least in HCV genotype 1b, which could leave IFN therapy less effective. However, the reason for the mutant specificity and genotype-tropism in coinfection is not clear. Further investigation at the molecular level is needed. Silent HBV DNA has been detected in hepatocellular carcinomas arising from anti-HCV positive cirrhosis [Koike et al., 1996; Urashima et al., 1997]. Thus, clarifying the relationship between serologically silent HBV and HCV is also important in order to establish the role of HBV in hepatocarcinogenesis of HCV-associated CLD.

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